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Vernonia galamensis and vernolic acid inhibit fatty acid biohydrogenation in vitro

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Abstract: Substituted long-chain fatty acids may be useful dietary supplements to suppress ruminal biohydrogenation of unsaturated fatty acids (UFA) and thereby increase the flow of UFA to meat and milk. The aim of this study was to determine if *Vernonia galamensis* (ironweed, a member of the sunflower family) and vernolic acid (cis-12,13-epoxy-cis-9-octadecenoic acid), the main constituent of the seed oil, affected the biohydrogenation of linoleic acid (LA; cis-9,cis-12-18:2) to rumenic acid (CLA; cis-9, trans-11-18:2), vaccenic acid (VA; trans-11-18:1) and stearic acid (SA; 18:0) by ruminal microorganisms. Ruminal digesta from four sheep receiving a mixed hay-concentrate diet were incubated in vitro with LA (0.2 g/L) □ 0.2 g/L vernolic acid or 5 g/L of dried flowers or leaves of *V. galamensis*, either alone or combined. Vernolic acid had a substantial effect on LA metabolism, causing decreases in cis-9, trans-11 CLA and VA accumulation as well as SA production ($P<0.05$). Vernolic acid inhibited growth of the rumen fatty acid-biohydrogenating bacterium, *Butyrivibrio fibrisolvens* but not *B. proteoclasticus* at 0.025 g/L; neither species grew at 0.05 g/L. An inhibition of the metabolism of LA as well as a decrease in the accumulation of cis-9, trans-11 CLA and a slowdown in its metabolism were observed in the presence of flowers, leaves and a combination of both ($P<0.05$). However, only incubations with flowers, either alone or with leaves, resulted in higher accumulation of VA ($P\leq 0.05$). Vernolic acid, constituted 2% of the total fatty acid content of flowers whereas it was not detected in leaves of *V. galamensis*. Thus, the greater accumulation of VA observed with flowers of *V. galamensis* was probably due to other components rather than vernolic acid. It was concluded that vernolic acid and *V. galamensis* inhibit the biohydrogenation of LA in vitro. *V. galamensis* could potentially be used as an additive to alter ruminal biohydrogenation, leading to greater concentrations of cis-9, trans-11 CLA in meat and milk. Evaluation of *V. galamensis* in vivo is required to confirm the present in vitro observations.

Ms. No. ANIFEE-16-7735R1 "Vernonia glauca and vernolic acid inhibit fatty acid biohydrogenation in vitro"

We are glad to know that our manuscript has been favorably received. In relation to the referee's comments, we have modified the manuscript accordingly. The changes made in the manuscript as well as the responses to the comments are detailed below (in red).

Reviewer #2: The authors have addressed all of the issues I raised in the initial review. I think the revised manuscript is interesting and provides new ideas about fatty acid biohydrogenation. The only comment of disagreement would be about the statement on line 47 and line 304. The authors can certainly speculate that the greater accumulation of VA observed with the flowers of *V. glauca* may have been due to the presence of other components. Yes, it is even possible that as yet unidentified compounds are present in *V. glauca*, and that these unidentified compounds are extremely potent even though they might be present at a very low concentration. However, the authors do not seem to have evidence that these putative "other" components are "present in higher amounts". I suggest they delete the statement "present in higher amounts".

AU: We agree with the reviewer and that statement has been deleted in the revised manuscript.

Reviewer #3: I have just read the revised version; I appreciate the authors' efforts to reply to my suggestions. The answers they gave are satisfactory.

In my opinion an item should be more stressed; as you said on lines 308-310 "Our present understanding of ruminal bacteria that biohydrogenate fatty acids is undoubtedly incomplete, because it is likely that certain members of the community have not yet been cultivated". This is a very important concept that you could emphasise by a sentence about it in the introduction of your paper; I would suggest on the line 60

AU: This information has been added to the revised manuscript (lines 65-68 "The most active biohydrogenating bacteria isolated from the rumen belong to *Butyrivibrio* group (Lourenco et al., 2010). However, other as yet uncultivated bacteria may play a more predominant role (Boeckaert et al., 2008; Huws et al., 2011)., as suggested.

Line 279: Replaced "Nobel" by "Noble"

Line 476: Replaced "CLA" by "VA"

Highlights

Vernonia galamensis and vernolic acid inhibit fatty acid biohydrogenation *in vitro*

Ramos Morales *et al*

- Substituted long-chain fatty acids inhibit ruminal fatty acid biohydrogenation
- We investigated the effects of *V. galamensis* and vernolic acid on biohydrogenation
- Both the plant and pure vernolic acid inhibited biohydrogenation by ruminal digesta
- Vernolic acid inhibited growth of the biohydrogenating bacteria in pure culture

1 *Vernonia galamensis* and vernolic acid inhibit fatty acid
2 biohydrogenation *in vitro*

3

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21 Abbreviations: CLA, conjugated linoleic acid; DHA, docosahexaenoic acid; DM, dry
22 matter; EPA, eicosapentaenoic acid; MS, mass spectrometry; LA, linoleic acid; LNA,
23 linolenic acid; SA, stearic acid; SRF, strained ruminal fluid; UFA, unsaturated fatty
24 acids; VA, vaccenic acid.

Abstract

Substituted long-chain fatty acids may be useful dietary supplements to suppress ruminal biohydrogenation of unsaturated fatty acids (UFA) and thereby increase the flow of UFA to meat and milk. The aim of this study was to determine if *Vernonia galamensis* (ironweed, a member of the sunflower family) and vernolic acid (*cis*-12,13-epoxy-*cis*-9-octadecenoic acid), the main constituent of the seed oil, affected the biohydrogenation of linoleic acid (LA; *cis*-9,*cis*-12-18:2) to rumenic acid (CLA; *cis*-9, *trans*-11-18:2), vaccenic acid (VA; *trans*-11-18:1) and stearic acid (SA; 18:0) by ruminal microorganisms. Ruminal digesta from four sheep receiving a mixed hay-concentrate diet were incubated *in vitro* with LA (0.2 g/L) \pm 0.2 g/L vernolic acid or 5 g/L of dried flowers or leaves of *V. galamensis*, either alone or combined. Vernolic acid had a substantial effect on LA metabolism, causing decreases in *cis*-9, *trans*-11 CLA and VA accumulation as well as SA production ($P < 0.05$). Vernolic acid inhibited growth of the rumen fatty acid-biohydrogenating bacterium, *Butyrivibrio fibrisolvens* but not *B. proteoclasticus* at 0.025 g/L; neither species grew at 0.05 g/L. An inhibition of the metabolism of LA as well as a decrease in the accumulation of *cis*-9, *trans*-11 CLA and a slowdown in its metabolism were observed in the presence of flowers, leaves and a combination of both ($P < 0.05$). However, only incubations with flowers, either alone or with leaves, resulted in higher accumulation of VA ($P \leq 0.05$). Vernolic acid, constituted 2% of the total fatty acid content of flowers whereas it was not detected in leaves of *V. galamensis*. Thus, the greater accumulation of VA observed with flowers of *V. galamensis* was probably due to other components rather than vernolic acid. It was concluded that vernolic acid and *V. galamensis* inhibit the biohydrogenation of LA *in vitro*. *V. galamensis* could potentially be used as an

additive to alter ruminal biohydrogenation, leading to greater concentrations of *cis*-9, *trans*-11 CLA in meat and milk. Evaluation of *V. galamensis* *in vivo* is required to confirm the present *in vitro* observations.

Keywords: biohydrogenation, conjugated linoleic acid, rumen, vernolic acid, *Vernonia galamensis*

1. Introduction

Ruminant products contain conjugated linoleic acids (CLA), of which the most abundant is usually rumenic acid (CLA; *cis*-9, *trans*-11-18:2), and vaccenic acid (VA; *trans*-11-18:1) which are potential health-promoting and disease-preventing agents (Kritchevsky, 2000; Field et al., 2009; Dilzer and Park, 2012). Conjugated linoleic acid and VA are produced as intermediate products in the biohydrogenation of linoleic acid (LA; *cis*-9, *cis*-12-18:2) present in the feed to stearic acid (SA; 18:0) (Jenkins et al., 2008). The most active biohydrogenating bacteria isolated from the rumen belong to *Butyrivibrio* group (Lourenco et al., 2010). However, other as yet uncultivated bacteria may play a more predominant role (Boeckaert et al., 2008; Huws et al., 2011). If ruminal biohydrogenation could be controlled by, for example, a dietary additive, the unsaturated fatty acid content, particularly *cis*-9, *trans*-11 CLA and VA, of ruminant products and their healthfulness would be improved.

Evidence is increasing that substituted long-chain fatty acids, comprised of a carbon chain with one or more substituent groups, including those from the plant kingdom (Durmic et al., 2008), may be effective feed additives to control biohydrogenation. Coronaric acid is a C-18 epoxy fatty acid that contributes to the inhibitory effect of

Chrysanthemum coronarium on biohydrogenation (Wood et al., 2010) that leads to an improved fatty acid composition of milk from sheep receiving *C. coronarium* (Cabiddu et al., 2006). Ricinoleic acid (12-hydroxy-*cis*-9-18:1), the main fatty acid component of castor oil, inhibited the biohydrogenation of LA *in vitro* (Ramos Morales et al., 2012). *Vernonia galamensis*, an East African plant of the sunflower family known sometimes as ironweed, contains a high concentration of vernolic acid (*cis*-12,13-epoxy-*cis*-9-18:1; 54-74%), an isomer of coronaric acid, which is present in the seed oil (Baye et al., 2005). The effects of neither the plant nor vernolic acid on fatty acid biohydrogenation have been investigated. The aim of the present experiments was to explore the potential of *V. galamensis* and vernolic acid to function as biohydrogenation inhibitors.

2. Material and methods

2.1. Animals and diets

Animal experimentation was carried out under conditions governed by a licence issued by the United Kingdom Home Office. Four mature sheep, each fitted with a ruminal cannula, received 800 g dry matter (DM)/day of ration comprising (g/kg DM) grass hay (300), rolled barley (422.5), soybean meal (167.5), molasses (100) and minerals and vitamins (10) as two equal meals (2×400 g) at 0800 and 1600. Samples of ruminal digesta were collected from each animal just before the morning feeding. Digesta samples were bubbled with CO₂, maintained at 39 °C, and strained ruminal fluid (SRF) was obtained by straining through double-layered muslin gauze.

Each set of incubations was carried out using ruminal digesta from four sheep incubated individually (four replicates).

2.2. *Vernonia galamensis* samples

Freeze dried samples of flowers and leaves of *V. galamensis* were obtained from the African Centre for Crop Improvement (University of KwaZulu-Natal, South Africa). Samples were ground to pass a 1 mm screen.

2.3. Incubations with ruminal digesta in vitro

In order to determine the effect of vernolic acid on the metabolism of LA, SRF was incubated with 0.2 g/L of LA (Sigma–Aldrich Co. Ltd., UK) in the presence or absence of vernolic acid (Larodan Fine Chemicals, Sweden) added at 0.2 g/L. Additionally, incubations of SRF with 0.2 g/L of either *cis*-9, *trans*-11 CLA or VA (Sigma–Aldrich Co. Ltd., UK), as substrates for the biohydrogenating bacteria, with or without vernolic acid (0.2 g/L), were carried out with the aim of studying where in the biohydrogenation sequence the inhibition by vernolic acid occurred. Likewise, in order to determine the effect of flowers or leaves of *V. galamensis* on the metabolism of LA, SRF was incubated with 0.2 g/L of LA in the presence or absence of 5 mg of ground and freeze dried flowers or leaves. Furthermore, in an attempt to study the possible synergistic effect between flowers and leaves on the metabolism of LA, incubations of SRF with LA (0.2 g/L) with or without a mixture of flowers and leaves (2.5 mg of each) of *V. galamensis* were carried out. Fatty acids were incubated as an oil in-water suspension obtained by sonication for about 4 min.

Tubes were removed at 0, 1, 3, 6, 9 and 24 h for fatty acid analysis. Reactions were stopped by heating in a heating block at 100°C for 10 min and tubes were stored at -20°C.

2.4. Incubations with pure cultures

Two species of ruminal bacteria were used. *Butyrivibrio fibrisolvens* JW11 was originally isolated from sheep as a proteolytic species (Wallace and Brammall, 1985). *Butyrivibrio proteoclasticus* P18 is a SA-producing bacterium isolated from grazing sheep (Wallace et al., 2006). These two species are the main cultivated species known to be involved in fatty acid biohydrogenation (Lourenço et al., 2010). *Propionibacterium acnes* G449 was isolated in the same study as *B. proteoclasticus* P18. *Megasphaera elsdenii* J1 was isolated from a sheep. These ruminal bacteria are held in the culture collection maintained at the Rowett Institute of Nutrition and Health.

In order to study the effect of vernolic acid on growth, incubations were carried out under O₂-free CO₂ at 39°C in Hungate-type tubes in the medium M2 (Hobson, 1969) without agar. Inoculum volumes were 5% (v/v) of a fresh overnight culture into 5 mL of medium. Vernolic acid was added to a final concentration of 0.0025, 0.025 and 0.05 g/L. Fatty acids were prepared as a separate solution, sonicated for 4 min in a small volume of medium and added to the medium before dispensing and autoclaving. Growth of bacteria was measured in triplicate from the increase in optical density at 650 nm of the control tubes using a Novaspec II spectrophotometer (Amersham Biosciences, UK).

2.5. Fatty acid extraction and analysis

Extraction of total fatty acids was based on the method of Folch et al. (1957), incorporating the modifications of Devillard et al. (2006). Nonadecanoic acid (0.1 mL of 0.2 g/L in methanol) was used as internal standard. Solid-phase extraction (Kalunzy et al., 1985) was used to separate free fatty acids from other lipids following Folch extraction.

Fatty acid methyl esters were prepared under mild conditions using methanolic H₂SO₄ (Wąsowska et al., 2006) as a catalyst and quantified using a gas chromatograph (model 6890, Agilent Technologies) equipped with a flame-ionization detector, quadrupole mass-selective detector (model 5973N) and a 100-m fused silica capillary column CP Sil 88 (i.d., 0.25 mm) coated with 0.2 µm film of cyanopropyl polysiloxane (Varian Analytical Instruments, Walton-on-Thames, Surrey, UK) using Helium as the carrier gas. The fatty acid methyl esters profile in 1 µl of sample at a split ratio of 20:1 was determined using a temperature gradient programme (initial temperature 80°C for 1 min; increased at a rate of 25°C/min to 160°C, which was held for 3 min, then increased at a rate of 1°C/min to 190°C, then further increased at a rate of 10°C/min to 230°C, held for 40 min). Injector and MS detector temperatures were maintained at 250 and 230°C, respectively. Peaks were routinely identified by comparison of retention times with authentic fatty acid methyl esters standard obtained from Sigma (Poole, Dorset, UK) and Matreya (Pleasant Gap, PA, USA). Identification was validated based on electron impact ionization spectra of fatty acid methyl esters obtained under an ionization voltage of 2247 eV.

Vernolic acid concentration in flowers and leaves was determined at the University of the Free State (South Africa). Fatty acids were extracted following the method of

Folch et al. (1957) and a base-catalysed transesterification with sodium methoxide was carried out as described by Park et al. (2001), Kramer et al. (2002) and Alfaia et al. (2007). Vernolic acid was determined using a Varian 430 flame ionization gas chromatograph with a fused silica capillary column Chrompack CP Sil 88 (100 m × 0.25 mm i.d. × 0.2 µm film thickness) and hydrogen as the carrier gas. Vernolic acid was determined using a temperature program that initially started at 40°C for 2 min, increased at a rate of 4°C/min to 230 which was held for 10 min. Injector and detector temperatures were maintained at 250°C.

2.6. Data Analysis

Mixed-culture data were analysed at each time point separately by randomized complete block ANOVA, with individual sheep as a blocking term, according to the model:

$$Y_{ijk} = \mu + D_i + A_j + e_{ijk}$$

Where Y_{ijk} is the dependent, continuous variable ($n = 4$), μ is the overall mean; D_i is the fixed effect of the treatment, A_j is the random effect of the animal inoculum ($j = 1$ to 4) and e_{ijk} is the residual error. Individual comparisons were determined by post hoc t -tests using the unprotected LSD. Pure-culture data were analysed by ANOVA, again compared at each sampling time. Genstat 10th edition (VSN International, UK) was used.

3. Results

3.1. Incubations of ruminal digesta with C-18 fatty acids and vernolic acid

199

200 *In vitro* incubations of SRF and LA either alone or with 0.2 g vernolic acid/L were
201 carried out in order to study the influence of vernolic acid on the metabolism of LA
202 (Fig. 1). An inhibition ($P<0.05$ at 1 and 3 h) of LA disappearance as well as an
203 inhibition of the accumulation of *cis*-9, *trans*-11-CLA ($P<0.05$ at 1, 3 and 6 h) and
204 VA ($P<0.05$) was observed in the presence of vernolic acid. Subsequently, SA
205 accumulation over time decreased ($P<0.05$) with vernolic acid.

206 Incubations of SRF with *cis*-9, *trans*-11 CLA as substrate (Fig. 2a) confirmed a
207 slightly slower metabolism compared to LA (Fig. 1a), as 29.6% and 60% of the initial
208 amount of CLA and LA added, respectively, disappeared after 1 h of the incubation.
209 Vaccenic acid accumulated and SA increased in much the same way as with LA.
210 Vernolic acid caused a slowdown of the loss of *cis*-9, *trans*-11 CLA ($P<0.05$ at 3 and
211 9 h), in comparison with the control incubations. Also, the accumulation of VA was
212 lower ($P<0.05$ at 1, 9 and 24 h), as was the increase of SA over time ($P<0.05$) in the
213 presence of vernolic acid (Fig. 2).

214 Vaccenic acid was metabolised more slowly than the dienoic acids, indeed its
215 concentration reached a plateau level at 12 h which was not changed at 24 h (Fig. 3).
216 Vernolic acid did not appear to impair the metabolism of VA; if anything the
217 percentage of VA that disappeared in the presence of vernolic acid was slightly
218 greater (38.5% vs. 30 and 39.8% vs. 29.5% at 9 and 24 h, respectively, $P<0.05$),
219 although less SA was produced over time as compared with the control ($P<0.05$).

220

221 3.2. Incubations of ruminal digesta with linoleic acid and leaves and flowers of
222 *Vernonia galamensis*.

223

Strained rumen fluid was incubated *in vitro* with LA (0.2 g/L) in the presence or absence of flowers or leaves of *V. galamensis* (Fig. 4). Incubations of *V. galamensis* leaves with LA led to an inhibition of the disappearance of LA ($P<0.05$) and lower accumulation of *cis*-9, *trans*-11-CLA ($P<0.001$ at 1 h) with slightly increased accumulation of VA and no effect on SA production. Incubations with flowers of *V. galamensis* and LA also resulted in an inhibition of disappearance of LA ($P<0.05$), although to a less extent than that observed with the leaves. A decrease in the accumulation of *cis*-9, *trans*-11 CLA was also shown in incubations with flowers ($P<0.001$ at 1 h), values being higher than those observed in the presence of leaves. However, VA concentration increased by 16-23% (VA tended to be higher, $P=0.054$ and 0.057 , at 1 and 9 h of the incubation, compared with the control). A similar effect, inhibition of the loss of LA ($P<0.05$) as well as decreased *cis*-9, *trans*-11 CLA accumulation ($P<0.05$ at 1 h) and increased accumulation of VA ($P=0.033$ at 1 h of the incubation), was observed when flowers and leaves were combined (Fig. 5), but no signs of synergy were evident. Oleic acid and LA were present at a higher concentration in flowers (0.618 and 2.92 mg/g DM) than leaves (0.367 and 2.08 mg/g DM). Also, the proportion of oleic and LA in the non-esterified form was higher in flowers than leaves (Table 1). Leaves of *V. galamensis* had a much higher concentration of linolenic acid (LNA; 13.9 mg/g DM) in comparison with flowers (1.98 mg/g DM). Vernolic acid, comprised 2% of the total fatty acids in flowers whereas it was not detected in leaves of *V. galamensis* (data not shown).

3.3. Influence of vernolic acid on growth of biohydrogenating ruminal bacteria

Vernolic acid did not inhibit growth of *B. fibrisolvens* or *B. proteoclasticus* at 0.0025 g/L ($P>0.05$; Fig. 6a). When added at 0.025 g/L, vernolic acid completely inhibited growth of *B. fibrisolvens* ($P<0.001$) while *B. proteoclasticus* grew after a lag time of 5 h (Fig. 6b). Neither species grew when vernolic acid was added at 0.5 g/L (Fig. 6c). Vernolic acid did not have any effect on *M. elsdenii* or *P. acnes* (not shown).

4. Discussion

Unsaturated fatty acids at high concentration inhibit their own biohydrogenation because they are toxic to the main species involved in biohydrogenation, namely those related to *Butyrivibrio* (Maia et al., 2007). The more unsaturated the fatty acid molecule, the greater seems to be its inhibitory effects (Maia et al., 2007). The fish oil fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are particularly effective (Fievez et al., 2003; AbuGhazaleh and Jenkins, 2004; Wąsowska et al., 2006; Maia et al., 2007); these fatty acids originate in algae, which has also shown to inhibit fatty acid biohydrogenation (Boeckaert et al., 2007; Vlaeminck et al., 2008; Toral et al., 2012). Few studies, however, have pointed out the potential of unsaturated hydroxy- and epoxy- fatty acids as manipulators of rumen biohydrogenation (Wood et al., 2010; Ramos-Morales et al., 2012).

Vernonia galamensis is a widely distributed weed in Eastern Africa, but a potential industrial oilseed crop. The seed oil of *V. galamensis* contains vernolic acid, a natural epoxy fatty acid that can replace the expensive synthetic epoxy compounds used in the plastic and coating industries. Our interest in vernolic acid stemmed from studies carried out with coronaric acid (Wood et al., 2010), an isomer of vernolic acid, which showed its effectiveness in inhibiting biohydrogenation. Also, we hypothesised that

273 the high LNA content in *V. galamensis* leaves reported by others (Baye et al., 2005)
274 could potentially have an inhibitory effect on fatty acid biohydrogenation.

275 The rapid loss of LA and transient accumulation of *cis*-9, *trans*-11 CLA and VA in
276 control incubations, followed by the slower accumulation of SA, are features that
277 have been observed previously (Wařowska et al., 2006; Ramos Morales et al., 2012).
278 They reflect the relative rates associated with the different steps in the pathway, viz.
279 LA >CLA>VA>SA (Noble et al., 1974). Vernolic acid slowed the disappearance of
280 LA and decreased the accumulation of *cis*-9, *trans*-11 CLA and VA and also the
281 increase in SA concentration. The inhibition of an accumulation of these
282 intermediates is therefore also reminiscent of previous studies with fish oil, EPA,
283 DHA and ricinoleic acid (Wařowska et al., 2006; Ramos Morales et al., 2012). From
284 these results, vernolic acid appears to be an inhibitor of LA metabolism and
285 biohydrogenation although it did not lead to the accumulation of *cis*-9, *trans*-11 CLA
286 or VA. This effect seems to be greater than that reported for coronaric acid (Wood et
287 al., 2010). It should be noted that Fig. 2 a) (also Fig. 3 a)) illustrates the metabolism
288 of esterified LA in the feed, for which the rate-limiting step will be lipolysis
289 (Lourenço et al., 2010). Vernolic acid did not appear to influence this rate,
290 presumably because it did not inhibit the rate-limiting step.

291 We hypothesised that flowers of *V. galamensis* would possibly have an inhibitory
292 effect on biohydrogenation due to their content in vernolic acid which has been found
293 to be present in seeds of *V. galamensis* (Baye et al., 2005). Our hypothesis is
294 consistent with previous work on the fatty acid composition of the seed oil of the
295 variety of *V. galamensis* used in our study (Shimelis et al. 2006). However, the
296 analysis of the fatty acid composition of the flowers revealed that vernolic acid
297 accounted for about 2% of total lipid (data not shown). The unexpected low vernolic

acid content in the flowers could be explained by differences in the mature stage of the seeds as it has been reported that seeds harvested at full maturity had significantly higher oil and vernolic content than comparable samples harvested at a less mature stage (Thompson et al., 1994). Also, it should be borne in mind that entire flowers rather than seeds were used in our incubations and thus the content of vernolic acid would have been lower than that of the seeds alone. Irrespective of the inhibitory effect on biohydrogenation shown in incubations with vernolic acid, the greater accumulation of VA observed with flowers of *V. galamensis* must have been due to other components rather than vernolic acid. Fatty acid analysis confirmed the discovery by Baye et al. (2005) that the leaves contained high concentrations of LNA, which would account for some inhibition of LA biohydrogenation (Maia et al., 2007). Our present understanding of ruminal bacteria that biohydrogenate fatty acids is undoubtedly incomplete, because it is likely that certain members of the community have not yet been cultivated (Boeckaert et al., 2008; Huws et al., 2011). Nonetheless, our best understanding is that members of the *B. fibrisolvens* group convert LA to VA via *cis*-9, *trans*-11-CLA, while *B. proteoclasticus* converts LA all the way to SA, also via *cis*-9, *trans*-11-CLA (Jenkins et al., 2008; Lourenço et al., 2010). Typically, *B. proteoclasticus* is more sensitive to the toxic effects of unsaturated fatty acids than *B. fibrisolvens* (Wallace et al., 2006; Ramos Morales et al., 2012); this is the first time that we have observed the opposite. *M. elsdenii* (Kim et al., 2002) and *P. acnes* (Devillard and Wallace, 2006) have both been implicated in the formation of *trans*-10, *cis*-12 CLA, with the latter being considered more likely to be the main contributor (Lourenço et al., 2010). They are important because *trans*-10, *cis*-12 CLA causes milk fat depression in lactating ruminants (Griinari et al., 1998; Lock et al., 2007).

4. Conclusions

Vernonia galamensis and vernolic acid inhibit LA biohydrogenation in vitro. Pure culture incubations with vernolic acid would suggest that this fatty acid may not be useful in preventing the formation of *trans*-10-18:1, which has been associated with milk fat depression in dairy cows. *Vernonia galamensis* could potentially be used as a manipulator of ruminal biohydrogenation, leading to greater concentrations of *cis*-9, *trans*-11 CLA in meat and milk. Further studies are required to confirm that *V. galamensis* alters biohydrogenation *in vivo*.

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References

346 AbuGhazaleh, A.A., Jenkins, T.C., 2004. Short communication: docosahexaenoic acid
 347 promotes vaccenic acid accumulation in mixed ruminal cultures when
 348 incubated with linoleic acid. J. Dairy Sci. 87, 1047-1050.

349 Alfaia, C.M.M, Castro, M.L.F., Martins, S.I.V., Portugal, A.P.V., Alves, S.P.A.,
 350 Fontes, C.M.G.A., Bessa, R.J.B., Prates, J.A.M., 2007. Effect of slaughter
 351 season on fatty acid composition, conjugated linoleic acid isomers and
 352 nutritional value of intramuscular fat in Barrosã-PDO veal. Meat Sci. 75, 44–52.

353 Baye, T., Becker, H.C., Witzke-Ehbrecht, S., 2005. *Vernonia galamensis*, a natural
 354 source of epoxy oil: variation in fatty acid composition of seed and leaf lipids.
 355 Ind. Crops Prod. 21, 257–26.

356 Boeckaert, C., Fievez, V., Van Hecke, D., Verstraete, W., Boon, N., 2007. Changes in
 357 rumen biohydrogenation intermediates and ciliate protozoa diversity after
 358 algae supplementation to dairy cattle. Eur. J. Lipid Sci. Technol. 109, 767-777.

359 Boeckaert, C., Vlaeminck, B., Fievez, V., Maignien, L., Dijkstra, J., Boon, N., 2008.
 360 Accumulation of trans C-18:1 fatty acids in the rumen after dietary algal
 361 supplementation is associated with changes in the *Butyrivibrio* community.
 362 Appl. Environ. Microbiol. 74, 6923-6930.

363 Cabiddu, A., Addis, M., Pinna, G., Spada, S., Fiori, M., Sitzia, M., Pirisi, A., Piredda,
 364 G., Molle, G., 2006. The inclusion of a daisy plant (*Chrysanthemum*
 365 *coronarium*) in dairy sheep diet. 1: Effect on milk and cheese fatty acid
 366 composition with particular reference to C18:2 *cis*-9, *trans*-11. Livestock Sci.
 367 101, 57-67.

368 Devillard, E., Wallace, R.J., 2006. Biohydrogenation in the rumen and human
 369 intestine: implications for CLA and PUFA. Lipid Technol. 18, 127-130.

370 Devillard, E., McIntosh, F.M., Newbold, C.J., Wallace, R.J., 2006. Rumen ciliate
 371 protozoa contain high concentrations of conjugated linoleic acids and vaccenic
 372 acid, yet do not hydrogenate linoleic acid or desaturate stearic acid. Br. J.
 373 Nutr. 96, 697-704.

374 Dilzer, A., Park, Y., 2012. Implication of conjugated linoleic acid (CLA) in human
 375 health. Crit. Rev. Food Sci. Nutr. 52, 488-513.

376 Durmic, Z., McSweeney, C.S., Kemp, K., Hutton, P., Wallace, R.J., Vercoe, P.E.,
 377 2008. Australian plants with potential to inhibit bacteria and processes
 378 involved in ruminal biohydrogenation of fatty acids. Anim. Feed Sci. Technol.
 379 145, 271-284.

380 Field, C.J., Blewett, H.H., Proctor, S., Vine, D., 2009. Human health benefits of
 381 vaccenic acid. Appl. Physiol. Nutr. Metab. 34, 979-991.

382 Fievez, V., Dohme, F., Danneels, M., Raes, K., Demeyer, D., 2003. Fish oils as potent
 383 rumen methane inhibitors and associated effects on rumen fermentation in
 384 vitro and in vivo. Anim. Feed Sci. Technol. 104, 41-58.

385 Folch, J., Lees, M., Soane, G.H., 1957. A simple method for the isolation and
 386 purification of total lipids from animal tissues. J. Biol. Chem. 226, 497-509.

387 Griinari, J.M., Dwyer, D.A., McGuire, M.A., Bauman, D.E., Palmquist, D.L.,
 388 Nurmela, K.V., 1998. Trans-octadecenoic acids and milk fat depression in
 389 lactating dairy cows. J. Dairy Sci. 81, 1251-1261.

390 Hobson, P.N., 1969. Rumen bacteria. Meth. Microbiol. 3B, 133-149.

391 Huws, S.A., Kim, E.J., Lee, M.R., Scott, M.B., Tweed, J.K., Pinloche, E., Wallace,
 392 R.J., Scollan, N.D., 2011. As yet uncultured bacteria phylogenetically
 393 classified as *Prevotella*, *Lachnospiraceae incertae sedis* and unclassified

394 *Bacteroidales*, *Clostridiales* and *Ruminococcaceae* may play a predominant
395 role in ruminal biohydrogenation. Environ. Microbiol. 13, 1500-1512.

396 Jenkins, T.C., Wallace, R.J., Moate, P.J., Mosley, E.E., 2008. Recent advances in
397 biohydrogenation of unsaturated fatty acids within the rumen microbial
398 ecosystem. J. Anim. Sci. 86, 397-412.

399 Kaluzny, M.A., Duncan, L.A., Merritt, M.V., Epps, D.E., 1985. Rapid separation of
400 lipid classes in high yield and purity using bonded phase columns. J. Lipid
401 Res. 26, 135-140.

402 Kramer, J. K., Blackadar, C.B., Zhou, J., 2002. Evaluation of two GC columns (60m
403 Supelcowax 10 and 100 m CP Sil 88) for analysis of milkfat with emphasis on
404 CLA, 18:1, 18:2 and 18:3 isomers, and short and long-chain fatty acids.
405 Lipids. 37, 823–835.

406 Kritchevsky, D., 2000. Antimutagenic and some other effects of conjugated linoleic
407 acid. Br. J. Nutr. 83, 459-465.

408 Kim, Y.J., Liu, R.H., Rychlik, J.L., Russell, J.B., 2002. The enrichment of a ruminal
409 bacterium (*Megasphaera elsdenii* YJ-4) that produces the *trans*-10, *cis*-12
410 isomer of conjugated linoleic acid. J. Appl. Microbiol. 92, 976-982.

411 Lock, A.L., Tyburczy, C., Dwyer, D.A., Harvatine, K.J., Destailats, F., Mouloungui,
412 Z., Candy, L., Bauman, D.E., 2007. *trans*-10 octadecenoic acid does not
413 reduce milk fat synthesis in dairy cows. J. Nutr. 137, 71-76.

414 Lourenço, M., Ramos-Morales, E., Wallace, R.J., 2010. The role of microbes in
415 rumen lipolysis and biohydrogenation and their manipulation. Animal 4, 1008-
416 1023.

417 Maia, M.R.G., Chaudhary, L.H., Figueres, L., Wallace, R.J., 2007. Metabolism of
 418 polyunsaturated fatty acids and their toxicity to the microflora of the rumen.
 419 Ant. V. Leeuwen. 91, 303-314.

420 Noble, R.C., Moore, J.H., Harfoot, C.G., 1974. Observations on the pattern on
 421 biohydrogenation of esterified and unesterified linoleic acid in the rumen. Br.
 422 J. Nutr. 31, 99-108.

423 Park, Y., Albright, K.J., Cai, Z.Y., Pariza, M.W., 2001. Comparison of methylation
 424 procedures for conjugated linoleic acid and artefact formation by commercial
 425 (trimethylsilyl) diazomethane. J. Agri. Food Chem. 49, 1158–1164.

426 Ramos Morales, E., Mata-Espinosa, M.A., McKain, N., Wallace, R.J., 2012.
 427 Ricinoleic acid inhibits methanogenesis and fatty acid biohydrogenation in
 428 ruminal digesta from sheep and in bacterial cultures. J. Anim. Sci. 90, 4943-
 429 4950.

430 Shimelis, H.A., Labuschagne, M.T., Hugo, A., 2006. Variation of oil content and fatty
 431 acid composition in selected lines of vernonia (*Vernonia galamensis* variety
 432 *ethiopica*). S. Afr. J. Plant Soil 23, 62-63

433 Thompson, A.E., Dierig, D.A., Kleiman, R., 1994. Variation in *Vernonia galamensis*
 434 flowering characteristics, seed oil and vernolic acid content. Ind. Crops Prod.
 435 3, 175-183.

436 Toral, P.G., Belenguer, A., Shingfield, K.J., Hervás, G., Toivonen, V., Frutos, P.,
 437 2012. Fatty acid composition and bacterial community changes in the rumen
 438 fluid of lactating sheep fed sunflower oil plus incremental levels of marine
 439 algae. J. Dairy Sci. 95, 794-806.

440 Vlaeminck, B., Mengistu, G., Fievez, V., de Jonge, L., Dijkstra, J., 2008. Effect of
 441 in vitro docosahexaenoic acid supplementation to marine algae-adapted and

442 unadapted rumen inoculum on the biohydrogenation of unsaturated fatty acids
 443 in freeze-dried grass. J. Dairy Sci. 91, 1122-1132.
 444 Wallace, R.J., Brammall, M.L., 1985. The role of different species of rumen bacteria
 445 in the hydrolysis of protein in the rumen. J. Gen. Microbiol. 131, 821–832.
 446 Wallace, R.J., Chaudhary, L.C., McKain, N., McEwan, N.R., Richardson, A.J.,
 447 Vercoe, P.E., Walker, N.D., Paillard, D., 2006. *Clostridium proteoclasticum*: a
 448 ruminal bacterium that forms stearic acid from linoleic acid. FEMS Microbiol.
 449 Lett. 265, 195-201.
 450 Wařowska, I., Maia, M.R.G., Niedzwiedzka, K.M., Czauderna, M., Ramalho Ribeiro,
 451 J.M.C., Devillard, E., Shingfield, K.J., Wallace, R.J., 2006. Influence of fish
 452 oil on ruminal biohydrogenation of C18 unsaturated fatty acids. Br. J. Nutr.
 453 95, 1199-1211.
 454 Wood, T.A., Ramos-Morales, E., McKain, N., Shen, X., Atasoglu, C., Wallace, R.J.
 455 2010. *Chrysanthemum coronarium* as a modulator of fatty acid
 456 biohydrogenation in the rumen. Anim. Feed Sci. Technol. 161, 28-37.

Figure captions

Figure 1. Influence of vernolic acid on metabolism of linoleic acid (LA) in ruminal fluid from sheep receiving a mixed grass hay/concentrate diet. Linoleic acid and vernolic acid were added to an initial concentration of 0.2 g/L. (a) LA. (b) *cis*-9, *trans*-11 CLA. (c) VA. (d) SA. Black symbols are from incubations with LA alone; open symbols are from incubations with LA + vernolic acid. Results are mean \pm SE from four sheep.

Figure 2. Influence of vernolic acid on metabolism of *cis*-9, *trans*-11 CLA in ruminal fluid from sheep receiving a mixed grass hay/concentrate diet. *cis*-9, *trans*-11 CLA and vernolic acid were added to an initial concentration of 0.2 g/L. (a) LA. (b) *cis*-9, *trans*-11 CLA. (c) VA. (d) SA. Black symbols are from incubations with *cis*-9, *trans*-11 CLA alone; open symbols are from incubations with *cis*-9, *trans*-11 CLA + vernolic acid. Results are mean \pm SE from four sheep.

Figure 3. Influence of vernolic acid on metabolism of VA in ruminal fluid from sheep receiving a mixed grass hay/concentrate diet. Vaccenic acid and vernolic acid were added to an initial concentration of 0.2 g/L. (a) LA. (b) VA. (d) SA. Black symbols are from incubations with VA alone; open symbols are from incubations with VA + vernolic acid. Results are mean \pm SE from four sheep.

Fig. 4. Influence of flowers or leaves of *V. galamensis* on metabolism of LA in ruminal fluid from sheep receiving a mixed grass hay/concentrate diet. LA was added to an initial concentration of 0.2 g/L and either flowers or leaves to 5 g/L. (a) LA. (b)

cis-9, *trans*-11 **CLA**. (c) VA. (d) SA. Black symbols are from incubations with LA alone, diagonally striped symbols are from incubations with LA + flowers, and open symbols are from incubations with LA + leaves. Results are mean \pm SE from 4 sheep.

Figure 5. Influence of a mixture of flowers and leaves of *V. galamensis* on metabolism of LA in ruminal fluid from sheep receiving a mixed grass hay/concentrate diet. LA was added to an initial concentration of 0.2 g/L and flowers and leaves were added at 2.5 g/L each. Results are mean \pm SE from four sheep. (a) LA. (b) *cis*-9, *trans*-11 **CLA**. (c) VA. (d) SA. Black symbols are from incubations with LA alone and open symbols are from incubations with LA and the mixture of flowers and leaves. Results are mean \pm SE from 4 sheep.

Figure 6. Influence of vernolic acid on growth of *B. fibrisolvens* JW11 (squares) and *B. proteoclasticus* P18 (triangles). Black symbols are from incubations of pure cultures with no fatty acid added; open symbols are from incubations with vernolic acid a) 0.0025 g/L. b) 0.025 g/L. c) 0.05 g/L. Results are means from three separate cultures. OD₆₅₀ is the optical density of the culture at 650 nm.

Table 1. Concentrations (mg/g DM) of oleic (*cis*-9-18:1), linoleic (*cis*-9, *cis*-12-18:2) and linolenic (*cis*-9,*cis*-12,*cis*-15-18:3) acids in total and non-esterified forms in samples of flowers and leaves of *Vernonia galamensis*.

	<i>cis</i> -9-18:1				<i>cis</i> -9, <i>cis</i> -12-18:2				<i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15-18:3			
					Non				Non			
	Non esterified		Total		esterified		Total		esterified		Total	
	Mean ^a	SE ^a	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Flowers	0.227	0.002	0.618	0.003	1.01	0.009	2.92	0.012	0.516	0.004	1.98	0.027
Leaves	0.035	0.001	0.367	0.004	0.203	0.001	2.08	0.031	0.865	0.008	13.9	0.183

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505 ^aMean and SE from three replicate analyses.

Author declaration template

We wish to confirm that there are no Known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of the authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

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1 *Vernonia galamensis* and vernolic acid inhibit fatty acid
2 biohydrogenation *in vitro*

3
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21 Abbreviations: CLA, conjugated linoleic acid; DHA, docosahexaenoic acid; DM, dry
22 matter; EPA, eicosapentaenoic acid; MS, mass spectrometry; LA, linoleic acid; LNA,
23 linolenic acid; SA, stearic acid; SRF, strained ruminal fluid; UFA, unsaturated fatty
24 acids; VA, vaccenic acid.

Abstract

Substituted long-chain fatty acids may be useful dietary supplements to suppress ruminal biohydrogenation of unsaturated fatty acids (UFA) and thereby increase the flow of UFA to meat and milk. The aim of this study was to determine if *Vernonia galamensis* (ironweed, a member of the sunflower family) and vernolic acid (*cis*-12,13-epoxy-*cis*-9-octadecenoic acid), the main constituent of the seed oil, affected the biohydrogenation of linoleic acid (LA; *cis*-9,*cis*-12-18:2) to rumenic acid (CLA; *cis*-9, *trans*-11-18:2), vaccenic acid (VA; *trans*-11-18:1) and stearic acid (SA; 18:0) by ruminal microorganisms. Ruminal digesta from four sheep receiving a mixed hay-concentrate diet were incubated *in vitro* with LA (0.2 g/L) \pm 0.2 g/L vernolic acid or 5 g/L of dried flowers or leaves of *V. galamensis*, either alone or combined. Vernolic acid had a substantial effect on LA metabolism, causing decreases in *cis*-9, *trans*-11 CLA and VA accumulation as well as SA production ($P < 0.05$). Vernolic acid inhibited growth of the rumen fatty acid-biohydrogenating bacterium, *Butyrivibrio fibrisolvens* but not *B. proteoclasticus* at 0.025 g/L; neither species grew at 0.05 g/L. An inhibition of the metabolism of LA as well as a decrease in the accumulation of *cis*-9, *trans*-11 CLA and a slowdown in its metabolism were observed in the presence of flowers, leaves and a combination of both ($P < 0.05$). However, only incubations with flowers, either alone or with leaves, resulted in higher accumulation of VA ($P \leq 0.05$). Vernolic acid, constituted 2% of the total fatty acid content of flowers whereas it was not detected in leaves of *V. galamensis*. Thus, the greater accumulation of VA observed with flowers of *V. galamensis* was probably due to other components rather than vernolic acid. It was concluded that vernolic acid and *V. galamensis* inhibit the biohydrogenation of LA *in vitro*. *V. galamensis* could potentially be used as an

additive to alter ruminal biohydrogenation, leading to greater concentrations of *cis*-9, *trans*-11 CLA in meat and milk. Evaluation of *V. galamensis in vivo* is required to confirm the present *in vitro* observations.

Keywords: biohydrogenation, conjugated linoleic acid, rumen, vernolic acid, *Vernonia galamensis*

1. Introduction

Ruminant products contain conjugated linoleic acids (CLA), of which the most abundant is usually rumenic acid (CLA; *cis*-9, *trans*-11-18:2), and vaccenic acid (VA; *trans*-11-18:1) which are potential health-promoting and disease-preventing agents (Kritchevsky, 2000; Field et al., 2009; Dilzer and Park, 2012). Conjugated linoleic acid and VA are produced as intermediate products in the biohydrogenation of linoleic acid (LA; *cis*-9, *cis*-12-18:2) present in the feed to stearic acid (SA; 18:0) (Jenkins et al., 2008). The most active biohydrogenating bacteria isolated from the rumen belong to Butyrivibrio group (Lourenco et al., 2010). However, other as yet uncultivated bacteria may play a more predominant role (Boeckaert et al., 2008; Huws et al., 2011). If ruminal biohydrogenation could be controlled by, for example, a dietary additive, the unsaturated fatty acid content, particularly *cis*-9, *trans*-11 CLA and VA, of ruminant products and their healthfulness would be improved.

Evidence is increasing that substituted long-chain fatty acids, comprised of a carbon chain with one or more substituent groups, including those from the plant kingdom (Durmic et al., 2008), may be effective feed additives to control biohydrogenation. Coronaric acid is a C-18 epoxy fatty acid that contributes to the inhibitory effect of

Chrysanthemum coronarium on biohydrogenation (Wood et al., 2010) that leads to an improved fatty acid composition of milk from sheep receiving *C. coronarium* (Cabiddu et al., 2006). Ricinoleic acid (12-hydroxy-*cis*-9-18:1), the main fatty acid component of castor oil, inhibited the biohydrogenation of LA *in vitro* (Ramos Morales et al., 2012). *Vernonia galamensis*, an East African plant of the sunflower family known sometimes as ironweed, contains a high concentration of vernolic acid (*cis*-12,13-epoxy-*cis*-9-18:1; 54-74%), an isomer of coronaric acid, which is present in the seed oil (Baye et al., 2005). The effects of neither the plant nor vernolic acid on fatty acid biohydrogenation have been investigated. The aim of the present experiments was to explore the potential of *V. galamensis* and vernolic acid to function as biohydrogenation inhibitors.

2. Material and methods

2.1. Animals and diets

Animal experimentation was carried out under conditions governed by a licence issued by the United Kingdom Home Office. Four mature sheep, each fitted with a ruminal cannula, received 800 g dry matter (DM)/day of ration comprising (g/kg DM) grass hay (300), rolled barley (422.5), soybean meal (167.5), molasses (100) and minerals and vitamins (10) as two equal meals (2×400 g) at 0800 and 1600. Samples of ruminal digesta were collected from each animal just before the morning feeding. Digesta samples were bubbled with CO₂, maintained at 39 °C, and strained ruminal fluid (SRF) was obtained by straining through double-layered muslin gauze.

Each set of incubations was carried out using ruminal digesta from four sheep incubated individually (four replicates).

2.2. *Vernonia galamensis* samples

Freeze dried samples of flowers and leaves of *V. galamensis* were obtained from the African Centre for Crop Improvement (University of KwaZulu-Natal, South Africa). Samples were ground to pass a 1 mm screen.

2.3. Incubations with ruminal digesta in vitro

In order to determine the effect of vernolic acid on the metabolism of LA, SRF was incubated with 0.2 g/L of LA (Sigma–Aldrich Co. Ltd., UK) in the presence or absence of vernolic acid (Larodan Fine Chemicals, Sweden) added at 0.2 g/L. Additionally, incubations of SRF with 0.2 g/L of either *cis*-9, *trans*-11 CLA or VA (Sigma–Aldrich Co. Ltd., UK), as substrates for the biohydrogenating bacteria, with or without vernolic acid (0.2 g/L), were carried out with the aim of studying where in the biohydrogenation sequence the inhibition by vernolic acid occurred. Likewise, in order to determine the effect of flowers or leaves of *V. galamensis* on the metabolism of LA, SRF was incubated with 0.2 g/L of LA in the presence or absence of 5 mg of ground and freeze dried flowers or leaves. Furthermore, in an attempt to study the possible synergistic effect between flowers and leaves on the metabolism of LA, incubations of SRF with LA (0.2 g/L) with or without a mixture of flowers and leaves (2.5 mg of each) of *V. galamensis* were carried out. Fatty acids were incubated as an oil in-water suspension obtained by sonication for about 4 min.

Tubes were removed at 0, 1, 3, 6, 9 and 24 h for fatty acid analysis. Reactions were stopped by heating in a heating block at 100°C for 10 min and tubes were stored at -20°C.

2.4. Incubations with pure cultures

Two species of ruminal bacteria were used. *Butyrivibrio fibrisolvens* JW11 was originally isolated from sheep as a proteolytic species (Wallace and Brammall, 1985). *Butyrivibrio proteoclasticus* P18 is a SA-producing bacterium isolated from grazing sheep (Wallace et al., 2006). These two species are the main cultivated species known to be involved in fatty acid biohydrogenation (Lourenço et al., 2010). *Propionibacterium acnes* G449 was isolated in the same study as *B. proteoclasticus* P18. *Megasphaera elsdenii* J1 was isolated from a sheep. These ruminal bacteria are held in the culture collection maintained at the Rowett Institute of Nutrition and Health.

In order to study the effect of vernolic acid on growth, incubations were carried out under O₂-free CO₂ at 39°C in Hungate-type tubes in the medium M2 (Hobson, 1969) without agar. Inoculum volumes were 5% (v/v) of a fresh overnight culture into 5 mL of medium. Vernolic acid was added to a final concentration of 0.0025, 0.025 and 0.05 g/L. Fatty acids were prepared as a separate solution, sonicated for 4 min in a small volume of medium and added to the medium before dispensing and autoclaving. Growth of bacteria was measured in triplicate from the increase in optical density at 650 nm of the control tubes using a Novaspec II spectrophotometer (Amersham Biosciences, UK).

2.5. Fatty acid extraction and analysis

Extraction of total fatty acids was based on the method of Folch et al. (1957), incorporating the modifications of Devillard et al. (2006). Nonadecanoic acid (0.1 mL of 0.2 g/L in methanol) was used as internal standard. Solid-phase extraction (Kalunzy et al., 1985) was used to separate free fatty acids from other lipids following Folch extraction.

Fatty acid methyl esters were prepared under mild conditions using methanolic H₂SO₄ (Wąsowska et al., 2006) as a catalyst and quantified using a gas chromatograph (model 6890, Agilent Technologies) equipped with a flame-ionization detector, quadrupole mass-selective detector (model 5973N) and a 100-m fused silica capillary column CP Sil 88 (i.d., 0.25 mm) coated with 0.2 µm film of cyanopropyl polysiloxane (Varian Analytical Instruments, Walton-on-Thames, Surrey, UK) using Helium as the carrier gas. The fatty acid methyl esters profile in 1 µl of sample at a split ratio of 20:1 was determined using a temperature gradient programme (initial temperature 80°C for 1 min; increased at a rate of 25°C/min to 160°C, which was held for 3 min, then increased at a rate of 1°C/min to 190°C, then further increased at a rate of 10°C/min to 230°C, held for 40 min). Injector and MS detector temperatures were maintained at 250 and 230°C, respectively. Peaks were routinely identified by comparison of retention times with authentic fatty acid methyl esters standard obtained from Sigma (Poole, Dorset, UK) and Matreya (Pleasant Gap, PA, USA). Identification was validated based on electron impact ionization spectra of fatty acid methyl esters obtained under an ionization voltage of 2247 eV.

Vernolic acid concentration in flowers and leaves was determined at the University of the Free State (South Africa). Fatty acids were extracted following the method of

Folch et al. (1957) and a base-catalysed transesterification with sodium methoxide was carried out as described by Park et al. (2001), Kramer et al. (2002) and Alfaia et al. (2007). Vernolic acid was determined using a Varian 430 flame ionization gas chromatograph with a fused silica capillary column Chrompack CP Sil 88 (100 m × 0.25 mm i.d. × 0.2 µm film thickness) and hydrogen as the carrier gas. Vernolic acid was determined using a temperature program that initially started at 40°C for 2 min, increased at a rate of 4°C/min to 230 which was held for 10 min. Injector and detector temperatures were maintained at 250°C.

2.6. Data Analysis

Mixed-culture data were analysed at each time point separately by randomized complete block ANOVA, with individual sheep as a blocking term, according to the model:

$$Y_{ijk} = \mu + D_i + A_j + e_{ijk}$$

Where Y_{ijk} is the dependent, continuous variable ($n = 4$), μ is the overall mean; D_i is the fixed effect of the treatment, A_j is the random effect of the animal inoculum ($j = 1$ to 4) and e_{ijk} is the residual error. Individual comparisons were determined by post hoc t -tests using the unprotected LSD. Pure-culture data were analysed by ANOVA, again compared at each sampling time. Genstat 10th edition (VSN International, UK) was used.

3. Results

3.1. Incubations of ruminal digesta with C-18 fatty acids and vernolic acid

199

200 *In vitro* incubations of SRF and LA either alone or with 0.2 g vernolic acid/L were
201 carried out in order to study the influence of vernolic acid on the metabolism of LA
202 (Fig. 1). An inhibition ($P<0.05$ at 1 and 3 h) of LA disappearance as well as an
203 inhibition of the accumulation of *cis*-9, *trans*-11-CLA ($P<0.05$ at 1, 3 and 6 h) and
204 VA ($P<0.05$) was observed in the presence of vernolic acid. Subsequently, SA
205 accumulation over time decreased ($P<0.05$) with vernolic acid.

206 Incubations of SRF with *cis*-9, *trans*-11 CLA as substrate (Fig. 2a) confirmed a
207 slightly slower metabolism compared to LA (Fig. 1a), as 29.6% and 60% of the initial
208 amount of CLA and LA added, respectively, disappeared after 1 h of the incubation.
209 Vaccenic acid accumulated and SA increased in much the same way as with LA.
210 Vernolic acid caused a slowdown of the loss of *cis*-9, *trans*-11 CLA ($P<0.05$ at 3 and
211 9 h), in comparison with the control incubations. Also, the accumulation of VA was
212 lower ($P<0.05$ at 1, 9 and 24 h), as was the increase of SA over time ($P<0.05$) in the
213 presence of vernolic acid (Fig. 2).

214 Vaccenic acid was metabolised more slowly than the dienoic acids, indeed its
215 concentration reached a plateau level at 12 h which was not changed at 24 h (Fig. 3).
216 Vernolic acid did not appear to impair the metabolism of VA; if anything the
217 percentage of VA that disappeared in the presence of vernolic acid was slightly
218 greater (38.5% vs. 30 and 39.8% vs. 29.5% at 9 and 24 h, respectively, $P<0.05$),
219 although less SA was produced over time as compared with the control ($P<0.05$).

220

221 3.2. Incubations of ruminal digesta with linoleic acid and leaves and flowers of
222 *Vernonia galamensis*.

223

Strained rumen fluid was incubated *in vitro* with LA (0.2 g/L) in the presence or absence of flowers or leaves of *V. galamensis* (Fig. 4). Incubations of *V. galamensis* leaves with LA led to an inhibition of the disappearance of LA ($P<0.05$) and lower accumulation of *cis*-9, *trans*-11-CLA ($P<0.001$ at 1 h) with slightly increased accumulation of VA and no effect on SA production. Incubations with flowers of *V. galamensis* and LA also resulted in an inhibition of disappearance of LA ($P<0.05$), although to a less extent than that observed with the leaves. A decrease in the accumulation of *cis*-9, *trans*-11 CLA was also shown in incubations with flowers ($P<0.001$ at 1 h), values being higher than those observed in the presence of leaves. However, VA concentration increased by 16-23% (VA tended to be higher, $P=0.054$ and 0.057 , at 1 and 9 h of the incubation, compared with the control). A similar effect, inhibition of the loss of LA ($P<0.05$) as well as decreased *cis*-9, *trans*-11 CLA accumulation ($P<0.05$ at 1 h) and increased accumulation of VA ($P=0.033$ at 1 h of the incubation), was observed when flowers and leaves were combined (Fig. 5), but no signs of synergy were evident. Oleic acid and LA were present at a higher concentration in flowers (0.618 and 2.92 mg/g DM) than leaves (0.367 and 2.08 mg/g DM). Also, the proportion of oleic and LA in the non-esterified form was higher in flowers than leaves (Table 1). Leaves of *V. galamensis* had a much higher concentration of linolenic acid (LNA; 13.9 mg/g DM) in comparison with flowers (1.98 mg/g DM). Vernolic acid, comprised 2% of the total fatty acids in flowers whereas it was not detected in leaves of *V. galamensis* (data not shown).

3.3. Influence of vernolic acid on growth of biohydrogenating ruminal bacteria

Vernolic acid did not inhibit growth of *B. fibrisolvens* or *B. proteoclasticus* at 0.0025 g/L ($P>0.05$; Fig. 6a). When added at 0.025 g/L, vernolic acid completely inhibited growth of *B. fibrisolvens* ($P<0.001$) while *B. proteoclasticus* grew after a lag time of 5 h (Fig. 6b). Neither species grew when vernolic acid was added at 0.5 g/L (Fig. 6c). Vernolic acid did not have any effect on *M. elsdenii* or *P. acnes* (not shown).

4. Discussion

Unsaturated fatty acids at high concentration inhibit their own biohydrogenation because they are toxic to the main species involved in biohydrogenation, namely those related to *Butyrivibrio* (Maia et al., 2007). The more unsaturated the fatty acid molecule, the greater seems to be its inhibitory effects (Maia et al., 2007). The fish oil fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are particularly effective (Fievez et al., 2003; AbuGhazaleh and Jenkins, 2004; Wąsowska et al., 2006; Maia et al., 2007); these fatty acids originate in algae, which has also shown to inhibit fatty acid biohydrogenation (Boeckaert et al., 2007; Vlaeminck et al., 2008; Toral et al., 2012). Few studies, however, have pointed out the potential of unsaturated hydroxy- and epoxy- fatty acids as manipulators of rumen biohydrogenation (Wood et al., 2010; Ramos-Morales et al., 2012).

Vernonia galamensis is a widely distributed weed in Eastern Africa, but a potential industrial oilseed crop. The seed oil of *V. galamensis* contains vernolic acid, a natural epoxy fatty acid that can replace the expensive synthetic epoxy compounds used in the plastic and coating industries. Our interest in vernolic acid stemmed from studies carried out with coronaric acid (Wood et al., 2010), an isomer of vernolic acid, which showed its effectiveness in inhibiting biohydrogenation. Also, we hypothesised that

273 the high LNA content in *V. galamensis* leaves reported by others (Baye et al., 2005)
274 could potentially have an inhibitory effect on fatty acid biohydrogenation.

275 The rapid loss of LA and transient accumulation of *cis*-9, *trans*-11 CLA and VA in
276 control incubations, followed by the slower accumulation of SA, are features that
277 have been observed previously (Wařowska et al., 2006; Ramos Morales et al., 2012).
278 They reflect the relative rates associated with the different steps in the pathway, *viz.*
279 LA >CLA>VA>SA (Noble et al., 1974). Vernolic acid slowed the disappearance of
280 LA and decreased the accumulation of *cis*-9, *trans*-11 CLA and VA and also the
281 increase in SA concentration. The inhibition of an accumulation of these
282 intermediates is therefore also reminiscent of previous studies with fish oil, EPA,
283 DHA and ricinoleic acid (Wařowska et al., 2006; Ramos Morales et al., 2012). From
284 these results, vernolic acid appears to be an inhibitor of LA metabolism and
285 biohydrogenation although it did not lead to the accumulation of *cis*-9, *trans*-11 CLA
286 or VA. This effect seems to be greater than that reported for coronaric acid (Wood et
287 al., 2010). It should be noted that Fig. 2 a) (also Fig. 3 a)) illustrates the metabolism
288 of esterified LA in the feed, for which the rate-limiting step will be lipolysis
289 (Lourenço et al., 2010). Vernolic acid did not appear to influence this rate,
290 presumably because it did not inhibit the rate-limiting step.

291 We hypothesised that flowers of *V. galamensis* would possibly have an inhibitory
292 effect on biohydrogenation due to their content in vernolic acid which has been found
293 to be present in seeds of *V. galamensis* (Baye et al., 2005). Our hypothesis is
294 consistent with previous work on the fatty acid composition of the seed oil of the
295 variety of *V. galamensis* used in our study (Shimelis et al. 2006). However, the
296 analysis of the fatty acid composition of the flowers revealed that vernolic acid
297 accounted for about 2% of total lipid (data not shown). The unexpected low vernolic

acid content in the flowers could be explained by differences in the mature stage of the seeds as it has been reported that seeds harvested at full maturity had significantly higher oil and vernolic content than comparable samples harvested at a less mature stage (Thompson et al., 1994). Also, it should be borne in mind that entire flowers rather than seeds were used in our incubations and thus the content of vernolic acid would have been lower than that of the seeds alone. Irrespective of the inhibitory effect on biohydrogenation shown in incubations with vernolic acid, the greater accumulation of VA observed with flowers of *V. galamensis* must have been due to other components rather than vernolic acid. Fatty acid analysis confirmed the discovery by Baye et al. (2005) that the leaves contained high concentrations of LNA, which would account for some inhibition of LA biohydrogenation (Maia et al., 2007). Our present understanding of ruminal bacteria that biohydrogenate fatty acids is undoubtedly incomplete, because it is likely that certain members of the community have not yet been cultivated (Boeckaert et al., 2008; Huws et al., 2011). Nonetheless, our best understanding is that members of the *B. fibrisolvens* group convert LA to VA via *cis*-9, *trans*-11-CLA, while *B. proteoclasticus* converts LA all the way to SA, also via *cis*-9, *trans*-11-CLA (Jenkins et al., 2008; Lourenço et al., 2010). Typically, *B. proteoclasticus* is more sensitive to the toxic effects of unsaturated fatty acids than *B. fibrisolvens* (Wallace et al., 2006; Ramos Morales et al., 2012); this is the first time that we have observed the opposite. *M. elsdenii* (Kim et al., 2002) and *P. acnes* (Devillard and Wallace, 2006) have both been implicated in the formation of *trans*-10, *cis*-12 CLA, with the latter being considered more likely to be the main contributor (Lourenço et al., 2010). They are important because *trans*-10, *cis*-12 CLA causes milk fat depression in lactating ruminants (Griinari et al., 1998; Lock et al., 2007).

4. Conclusions

Vernonia galamensis and vernolic acid inhibit LA biohydrogenation in vitro. Pure culture incubations with vernolic acid would suggest that this fatty acid may not be useful in preventing the formation of *trans*-10-18:1, which has been associated with milk fat depression in dairy cows. *Vernonia galamensis* could potentially be used as a manipulator of ruminal biohydrogenation, leading to greater concentrations of *cis*-9, *trans*-11 CLA in meat and milk. Further studies are required to confirm that *V. galamensis* alters biohydrogenation *in vivo*.

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References

346 AbuGhazaleh, A.A., Jenkins, T.C., 2004. Short communication: docosaheptaenoic acid
 347 promotes vaccenic acid accumulation in mixed ruminal cultures when
 348 incubated with linoleic acid. J. Dairy Sci. 87, 1047-1050.

349 Alfaia, C.M.M, Castro, M.L.F., Martins, S.I.V., Portugal, A.P.V., Alves, S.P.A.,
 350 Fontes, C.M.G.A., Bessa, R.J.B., Prates, J.A.M., 2007. Effect of slaughter
 351 season on fatty acid composition, conjugated linoleic acid isomers and
 352 nutritional value of intramuscular fat in Barrosã-PDO veal. Meat Sci. 75, 44–52.

353 Baye, T., Becker, H.C., Witzke-Ehbrecht, S., 2005. *Vernonia galamensis*, a natural
 354 source of epoxy oil: variation in fatty acid composition of seed and leaf lipids.
 355 Ind. Crops Prod. 21, 257–26.

356 Boeckaert, C., Fievez, V., Van Hecke, D., Verstraete, W., Boon, N., 2007. Changes in
 357 rumen biohydrogenation intermediates and ciliate protozoa diversity after
 358 algae supplementation to dairy cattle. Eur. J. Lipid Sci. Technol. 109, 767-777.

359 Boeckaert, C., Vlaeminck, B., Fievez, V., Maignien, L., Dijkstra, J., Boon, N., 2008.
 360 Accumulation of trans C-18:1 fatty acids in the rumen after dietary algal
 361 supplementation is associated with changes in the *Butyrivibrio* community.
 362 Appl. Environ. Microbiol. 74, 6923-6930.

363 Cabiddu, A., Addis, M., Pinna, G., Spada, S., Fiori, M., Sitzia, M., Pirisi, A., Piredda,
 364 G., Molle, G., 2006. The inclusion of a daisy plant (*Chrysanthemum*
 365 *coronarium*) in dairy sheep diet. 1: Effect on milk and cheese fatty acid
 366 composition with particular reference to C18:2 *cis*-9, *trans*-11. Livestock Sci.
 367 101, 57-67.

368 Devillard, E., Wallace, R.J., 2006. Biohydrogenation in the rumen and human
 369 intestine: implications for CLA and PUFA. Lipid Technol. 18, 127-130.

370 Devillard, E., McIntosh, F.M., Newbold, C.J., Wallace, R.J., 2006. Rumen ciliate
 371 protozoa contain high concentrations of conjugated linoleic acids and vaccenic
 372 acid, yet do not hydrogenate linoleic acid or desaturate stearic acid. Br. J.
 373 Nutr. 96, 697-704.

374 Dilzer, A., Park, Y., 2012. Implication of conjugated linoleic acid (CLA) in human
 375 health. Crit. Rev. Food Sci. Nutr. 52, 488-513.

376 Durmic, Z., McSweeney, C.S., Kemp, K., Hutton, P., Wallace, R.J., Vercoe, P.E.,
 377 2008. Australian plants with potential to inhibit bacteria and processes
 378 involved in ruminal biohydrogenation of fatty acids. Anim. Feed Sci. Technol.
 379 145, 271-284.

380 Field, C.J., Blewett, H.H., Proctor, S., Vine, D., 2009. Human health benefits of
 381 vaccenic acid. Appl. Physiol. Nutr. Metab. 34, 979-991.

382 Fievez, V., Dohme, F., Danneels, M., Raes, K., Demeyer, D., 2003. Fish oils as potent
 383 rumen methane inhibitors and associated effects on rumen fermentation in
 384 vitro and in vivo. Anim. Feed Sci. Technol. 104, 41-58.

385 Folch, J., Lees, M., Soane, G.H., 1957. A simple method for the isolation and
 386 purification of total lipids from animal tissues. J. Biol. Chem. 226, 497-509.

387 Griinari, J.M., Dwyer, D.A., McGuire, M.A., Bauman, D.E., Palmquist, D.L.,
 388 Nurmela, K.V., 1998. Trans-octadecenoic acids and milk fat depression in
 389 lactating dairy cows. J. Dairy Sci. 81, 1251-1261.

390 Hobson, P.N., 1969. Rumen bacteria. Meth. Microbiol. 3B, 133-149.

391 Huws, S.A., Kim, E.J., Lee, M.R., Scott, M.B., Tweed, J.K., Pinloche, E., Wallace,
 392 R.J., Scollan, N.D., 2011. As yet uncultured bacteria phylogenetically
 393 classified as *Prevotella*, *Lachnospiraceae incertae sedis* and unclassified

394 *Bacteroidales*, *Clostridiales* and *Ruminococcaceae* may play a predominant
395 role in ruminal biohydrogenation. Environ. Microbiol. 13, 1500-1512.

396 Jenkins, T.C., Wallace, R.J., Moate, P.J., Mosley, E.E., 2008. Recent advances in
397 biohydrogenation of unsaturated fatty acids within the rumen microbial
398 ecosystem. J. Anim. Sci. 86, 397-412.

399 Kaluzny, M.A., Duncan, L.A., Merritt, M.V., Epps, D.E., 1985. Rapid separation of
400 lipid classes in high yield and purity using bonded phase columns. J. Lipid
401 Res. 26, 135-140.

402 Kramer, J. K., Blackadar, C.B., Zhou, J., 2002. Evaluation of two GC columns (60m
403 Supelcowax 10 and 100 m CP Sil 88) for analysis of milkfat with emphasis on
404 CLA, 18:1, 18:2 and 18:3 isomers, and short and long-chain fatty acids.
405 Lipids. 37, 823–835.

406 Kritchevsky, D., 2000. Antimutagenic and some other effects of conjugated linoleic
407 acid. Br. J. Nutr. 83, 459-465.

408 Kim, Y.J., Liu, R.H., Rychlik, J.L., Russell, J.B., 2002. The enrichment of a ruminal
409 bacterium (*Megasphaera elsdenii* YJ-4) that produces the *trans*-10, *cis*-12
410 isomer of conjugated linoleic acid. J. Appl. Microbiol. 92, 976-982.

411 Lock, A.L., Tyburczy, C., Dwyer, D.A., Harvatine, K.J., Destailats, F., Mouloungui,
412 Z., Candy, L., Bauman, D.E., 2007. *trans*-10 octadecenoic acid does not
413 reduce milk fat synthesis in dairy cows. J. Nutr. 137, 71-76.

414 Lourenço, M., Ramos-Morales, E., Wallace, R.J., 2010. The role of microbes in
415 rumen lipolysis and biohydrogenation and their manipulation. Animal 4, 1008-
416 1023.

417 Maia, M.R.G., Chaudhary, L.H., Figueres, L., Wallace, R.J., 2007. Metabolism of
 418 polyunsaturated fatty acids and their toxicity to the microflora of the rumen.
 419 Ant. V. Leeuwen. 91, 303-314.

420 Noble, R.C., Moore, J.H., Harfoot, C.G., 1974. Observations on the pattern on
 421 biohydrogenation of esterified and unesterified linoleic acid in the rumen. Br.
 422 J. Nutr. 31, 99-108.

423 Park, Y., Albright, K.J., Cai, Z.Y., Pariza, M.W., 2001. Comparison of methylation
 424 procedures for conjugated linoleic acid and artefact formation by commercial
 425 (trimethylsilyl) diazomethane. J. Agri. Food Chem. 49, 1158–1164.

426 Ramos Morales, E., Mata-Espinosa, M.A., McKain, N., Wallace, R.J., 2012.
 427 Ricinoleic acid inhibits methanogenesis and fatty acid biohydrogenation in
 428 ruminal digesta from sheep and in bacterial cultures. J. Anim. Sci. 90, 4943-
 429 4950.

430 Shimelis, H.A., Labuschagne, M.T., Hugo, A., 2006. Variation of oil content and fatty
 431 acid composition in selected lines of vernonia (*Vernonia galamensis* variety
 432 *ethiopica*). S. Afr. J. Plant Soil 23, 62-63

433 Thompson, A.E., Dierig, D.A., Kleiman, R., 1994. Variation in *Vernonia galamensis*
 434 flowering characteristics, seed oil and vernolic acid content. Ind. Crops Prod.
 435 3, 175-183.

436 Toral, P.G., Belenguer, A., Shingfield, K.J., Hervas, G., Toivonen, V., Frutos, P.,
 437 2012. Fatty acid composition and bacterial community changes in the rumen
 438 fluid of lactating sheep fed sunflower oil plus incremental levels of marine
 439 algae. J. Dairy Sci. 95, 794-806.

440 Vlaeminck, B., Mengistu, G., Fievez, V., de Jonge, L., Dijkstra, J., 2008. Effect of
 441 in vitro docosahexaenoic acid supplementation to marine algae-adapted and

442 unadapted rumen inoculum on the biohydrogenation of unsaturated fatty acids
 443 in freeze-dried grass. J. Dairy Sci. 91, 1122-1132.
 444 Wallace, R.J., Brammall, M.L., 1985. The role of different species of rumen bacteria
 445 in the hydrolysis of protein in the rumen. J. Gen. Microbiol. 131, 821–832.
 446 Wallace, R.J., Chaudhary, L.C., McKain, N., McEwan, N.R., Richardson, A.J.,
 447 Vercoe, P.E., Walker, N.D., Paillard, D., 2006. *Clostridium proteoclasticum*: a
 448 ruminal bacterium that forms stearic acid from linoleic acid. FEMS Microbiol.
 449 Lett. 265, 195-201.
 450 Wařowska, I., Maia, M.R.G., Niedzwiedzka, K.M., Czauderna, M., Ramalho Ribeiro,
 451 J.M.C., Devillard, E., Shingfield, K.J., Wallace, R.J., 2006. Influence of fish
 452 oil on ruminal biohydrogenation of C18 unsaturated fatty acids. Br. J. Nutr.
 453 95, 1199-1211.
 454 Wood, T.A., Ramos-Morales, E., McKain, N., Shen, X., Atasoglu, C., Wallace, R.J.
 455 2010. *Chrysanthemum coronarium* as a modulator of fatty acid
 456 biohydrogenation in the rumen. Anim. Feed Sci. Technol. 161, 28-37.

Figure captions

Figure 1. Influence of vernolic acid on metabolism of linoleic acid (LA) in ruminal fluid from sheep receiving a mixed grass hay/concentrate diet. Linoleic acid and vernolic acid were added to an initial concentration of 0.2 g/L. (a) LA. (b) *cis*-9, *trans*-11 CLA. (c) VA. (d) SA. Black symbols are from incubations with LA alone; open symbols are from incubations with LA + vernolic acid. Results are mean \pm SE from four sheep.

Figure 2. Influence of vernolic acid on metabolism of *cis*-9, *trans*-11 CLA in ruminal fluid from sheep receiving a mixed grass hay/concentrate diet. *cis*-9, *trans*-11 CLA and vernolic acid were added to an initial concentration of 0.2 g/L. (a) LA. (b) *cis*-9, *trans*-11 CLA. (c) VA. (d) SA. Black symbols are from incubations with *cis*-9, *trans*-11 CLA alone; open symbols are from incubations with *cis*-9, *trans*-11 CLA + vernolic acid. Results are mean \pm SE from four sheep.

Figure 3. Influence of vernolic acid on metabolism of VA in ruminal fluid from sheep receiving a mixed grass hay/concentrate diet. Vaccenic acid and vernolic acid were added to an initial concentration of 0.2 g/L. (a) LA. (b) VA. (d) SA. Black symbols are from incubations with VA alone; open symbols are from incubations with VA + vernolic acid. Results are mean \pm SE from four sheep.

Fig. 4. Influence of flowers or leaves of *V. galamensis* on metabolism of LA in ruminal fluid from sheep receiving a mixed grass hay/concentrate diet. LA was added to an initial concentration of 0.2 g/L and either flowers or leaves to 5 g/L. (a) LA. (b)

cis-9, *trans*-11 CLA. (c) VA. (d) SA. Black symbols are from incubations with LA alone, diagonally striped symbols are from incubations with LA + flowers, and open symbols are from incubations with LA + leaves. Results are mean \pm SE from 4 sheep.

Figure 5. Influence of a mixture of flowers and leaves of *V. galamensis* on metabolism of LA in ruminal fluid from sheep receiving a mixed grass hay/concentrate diet. LA was added to an initial concentration of 0.2 g/L and flowers and leaves were added at 2.5 g/L each. Results are mean \pm SE from four sheep. (a) LA. (b) *cis*-9, *trans*-11 CLA. (c) VA. (d) SA. Black symbols are from incubations with LA alone and open symbols are from incubations with LA and the mixture of flowers and leaves. Results are mean \pm SE from 4 sheep.

Figure 6. Influence of vernolic acid on growth of *B. fibrisolvens* JW11 (squares) and *B. proteoclasticus* P18 (triangles). Black symbols are from incubations of pure cultures with no fatty acid added; open symbols are from incubations with vernolic acid a) 0.0025 g/L. b) 0.025 g/L. c) 0.05 g/L. Results are means from three separate cultures. OD₆₅₀ is the optical density of the culture at 650 nm.

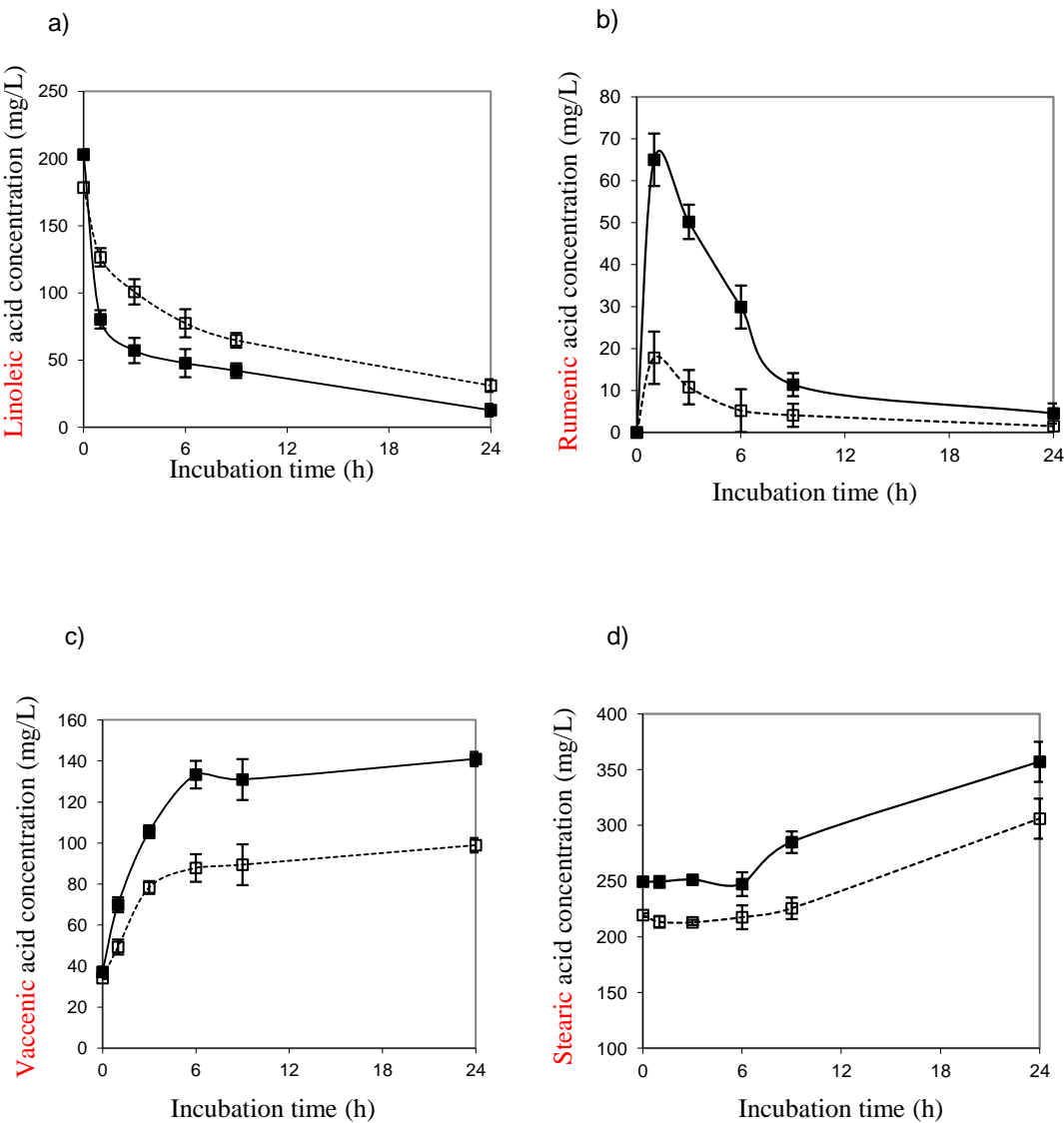
Table 1. Concentrations (mg/g DM) of oleic (*cis*-9-18:1), linoleic (*cis*-9, *cis*-12-18:2) and linolenic (*cis*-9,*cis*-12,*cis*-15-18:3) acids in total and non-esterified forms in samples of flowers and leaves of *Vernonia galamensis*.

	<i>cis</i> -9-18:1				<i>cis</i> -9, <i>cis</i> -12-18:2				<i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15-18:3			
					Non				Non			
	Non esterified		Total		esterified		Total		esterified		Total	
	Mean ^a	SE ^a	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Flowers	0.227	0.002	0.618	0.003	1.01	0.009	2.92	0.012	0.516	0.004	1.98	0.027
Leaves	0.035	0.001	0.367	0.004	0.203	0.001	2.08	0.031	0.865	0.008	13.9	0.183

504

505 ^aMean and SE from three replicate analyses.

1



2 **Figure 1. Ramos Morales et al.**

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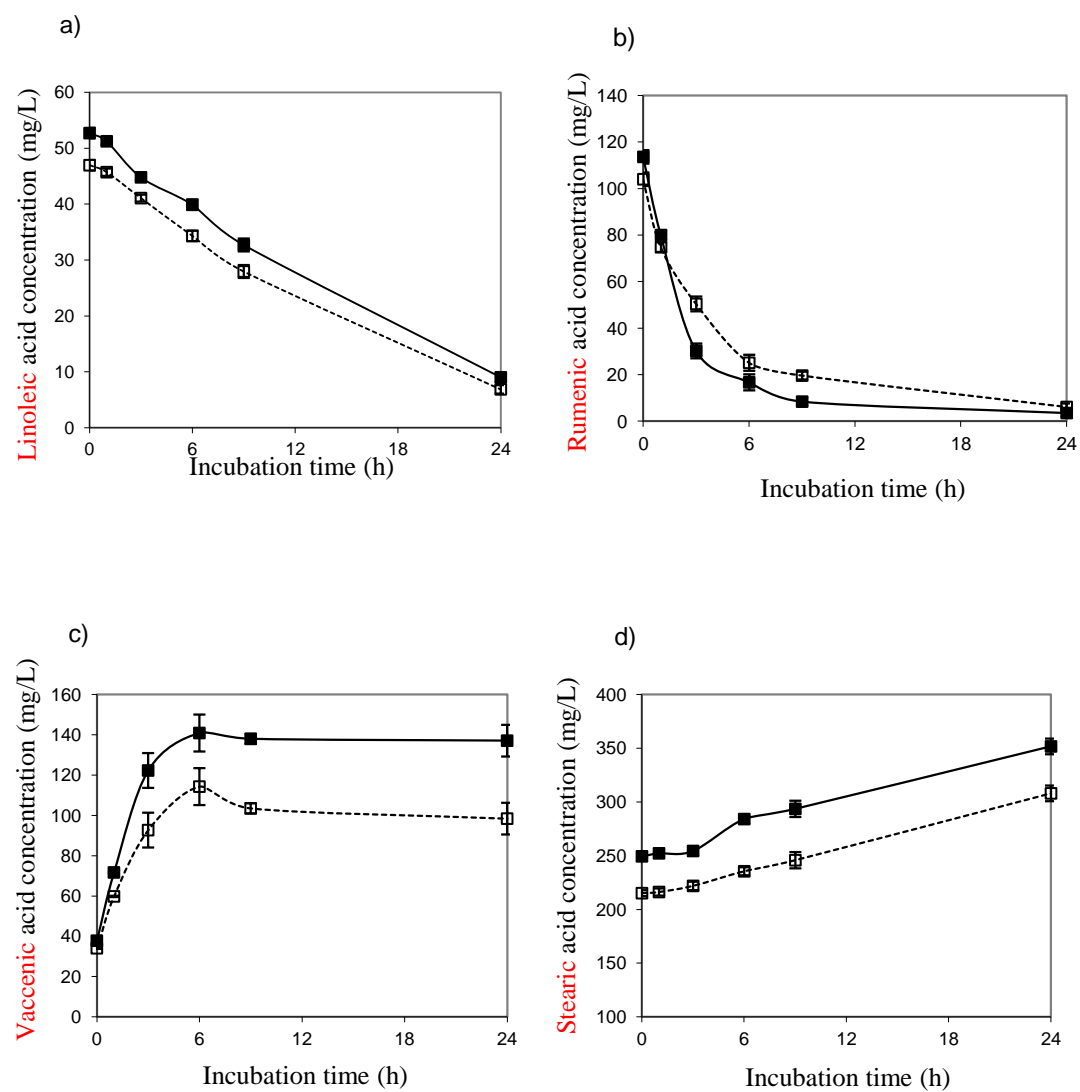
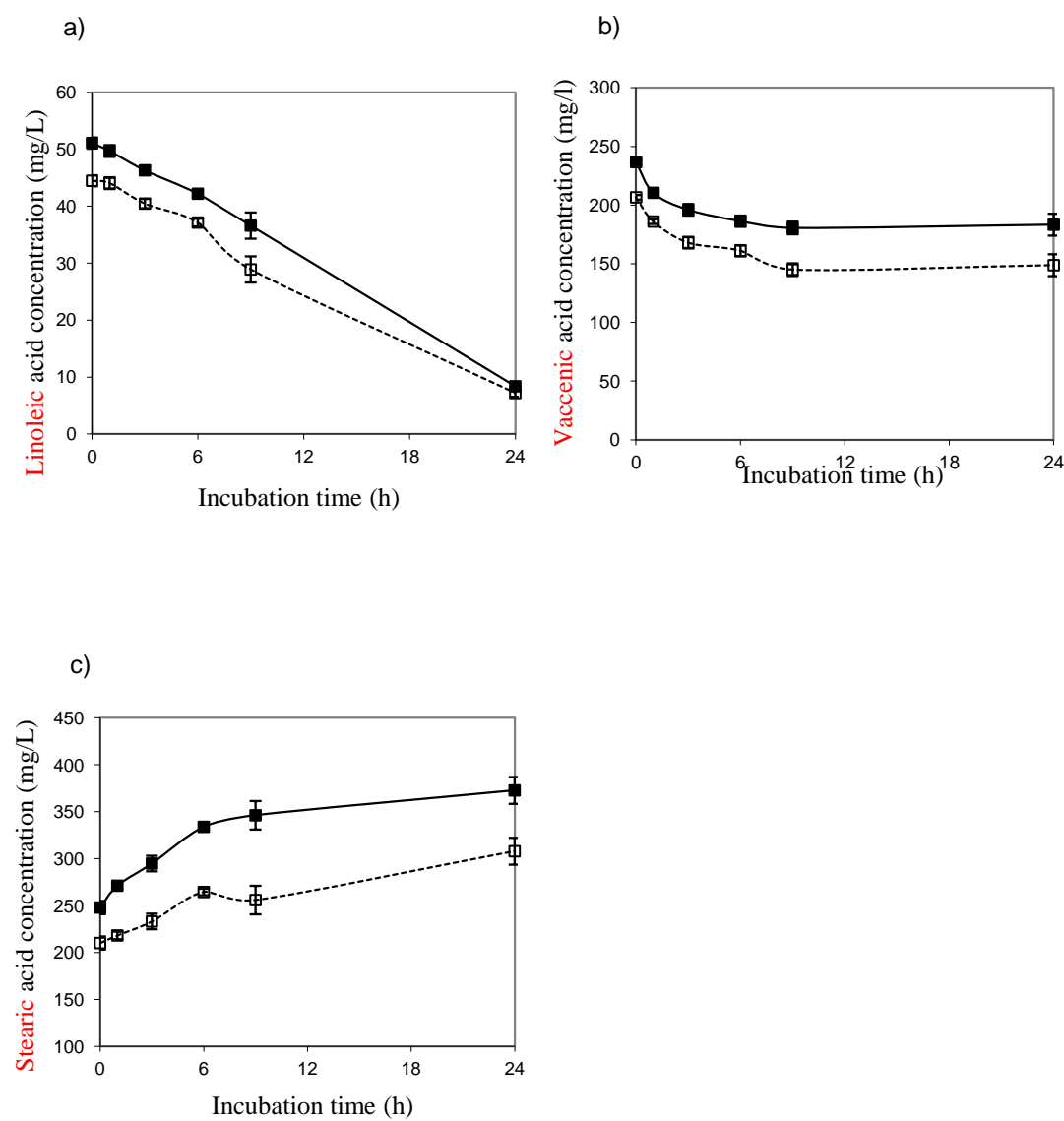


Figure 2. Ramos Morales et al.

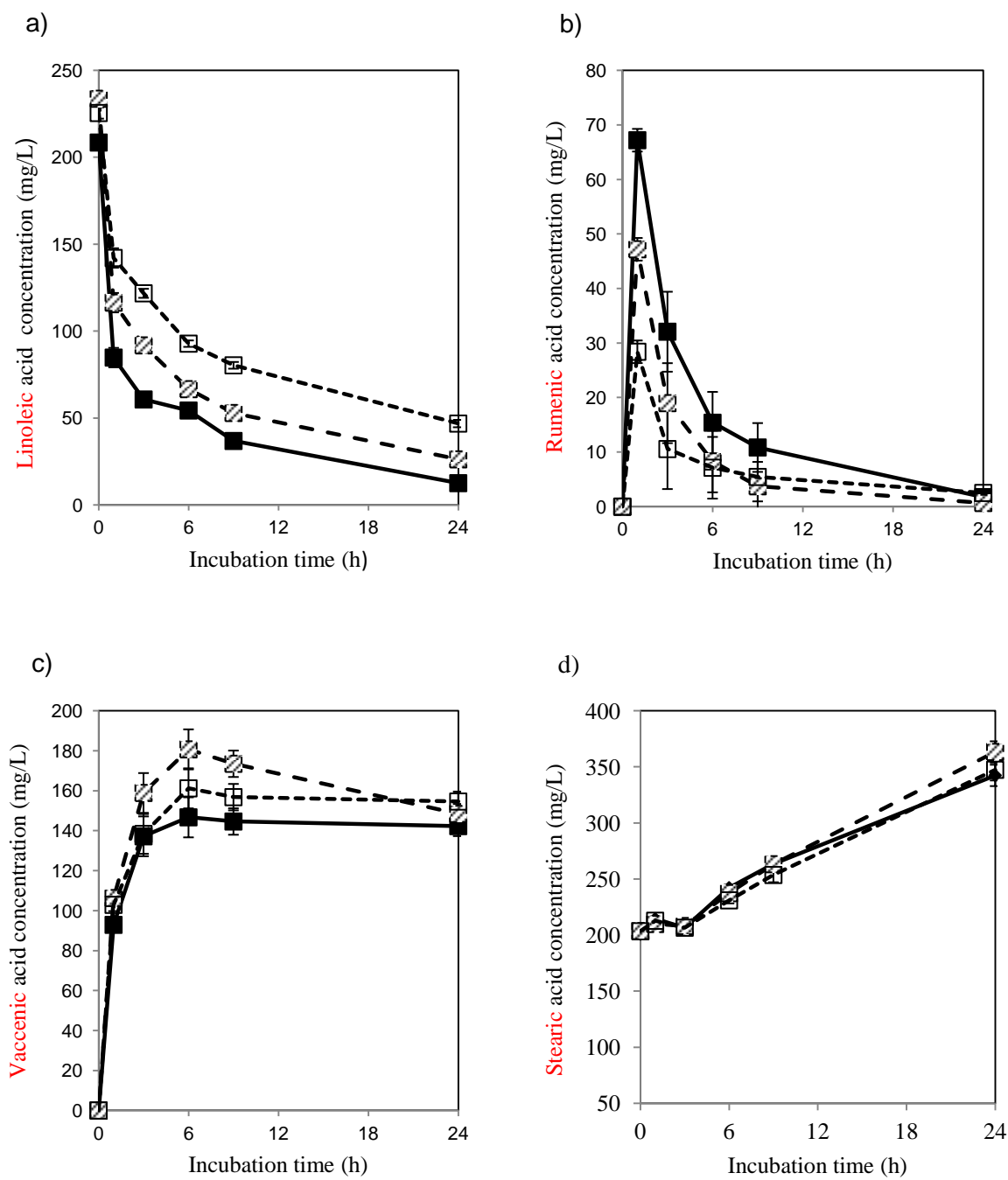
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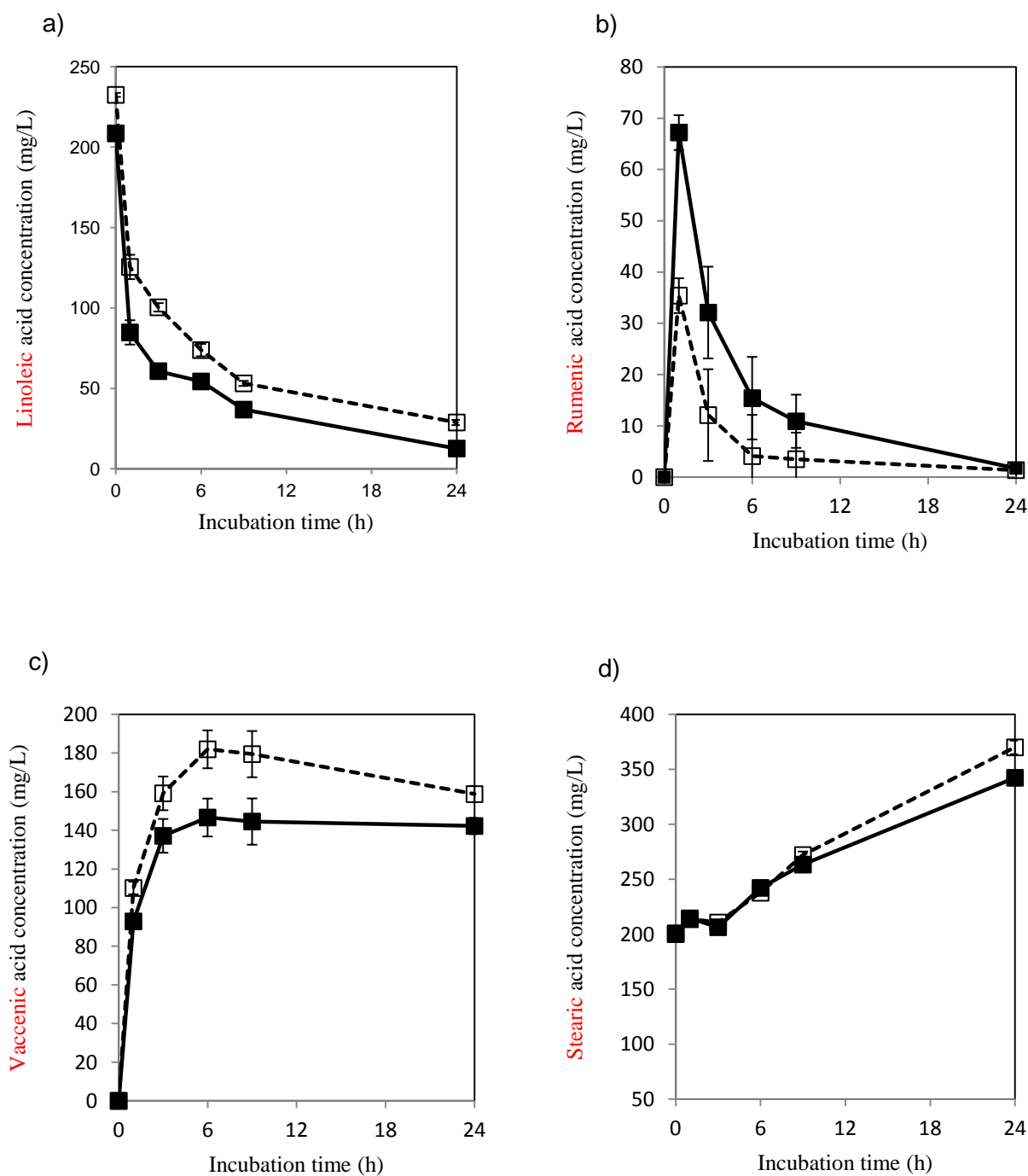


2 **Figure 3. Ramos Morales et al.**

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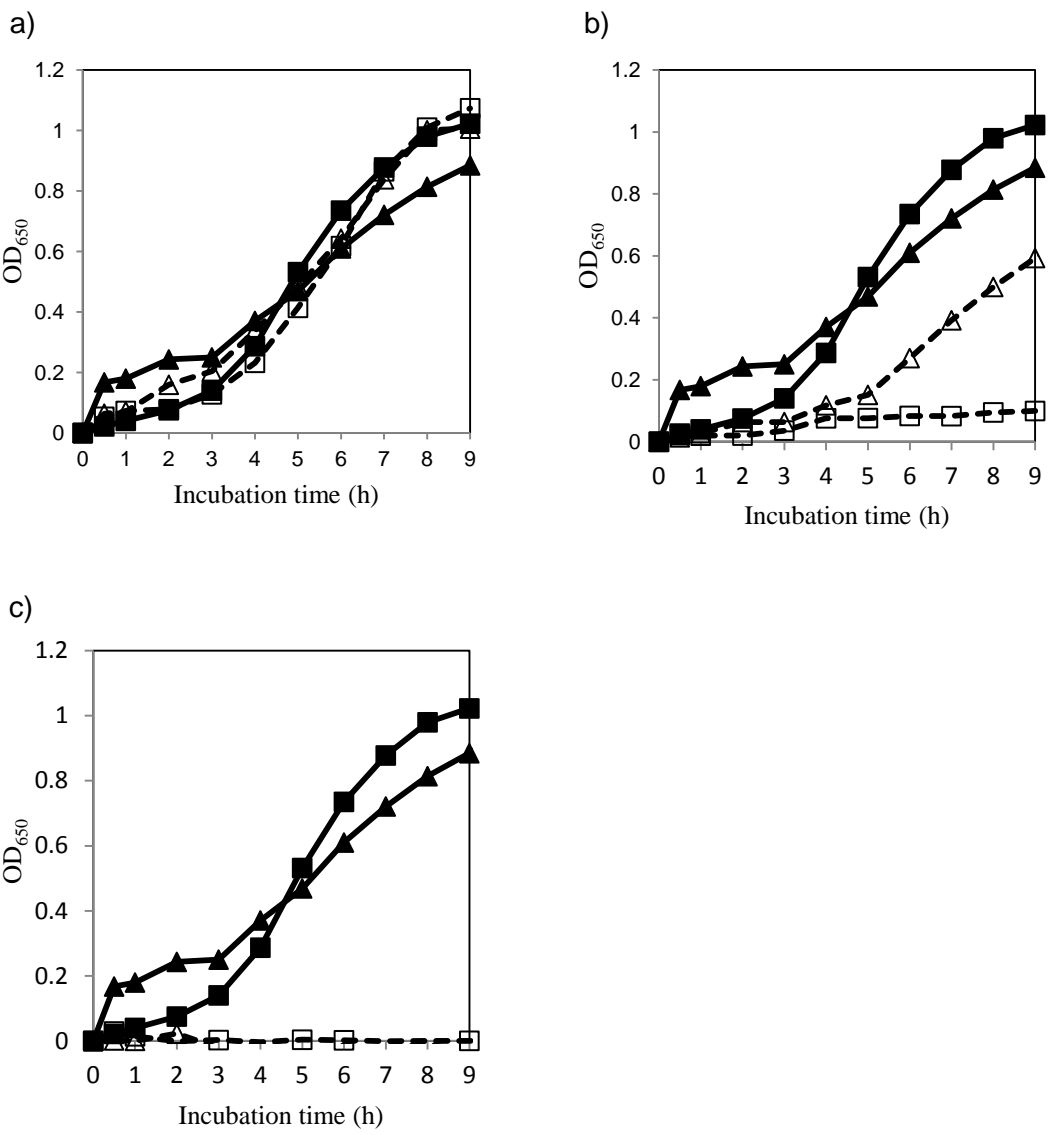


2 **Figure 4. Ramos Morales et al.**



1 **Figure 5. Ramos Morales et al.**

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2 **Figure 6. Ramos Morales et al.**